Detection and Estimation of Steroidal Sapogenins in Plant Tissue

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In the course of screening hundreds of plant samples for steroidal sapogenins, it became imperative to find a procedure that would rapidly detect and estimate these compounds. A literature search indicated that a rapid and specific procedure was not available. It was found that a negative hemolytic test on alcoholic plant extracts was definite proof of the absence of steroidal saponins. Samples giving positive hemolytic tests were acid hydrolyzed and acetylated, yielding a crude residue. Steroidal sapogenins could be detected, and the quantity present estimated from the highly specific infrared absorption spectra of all sapogenins. By this procedure, a large number of plant samples (40 to 50 by two workers in a 40-hour week) can be screened rapidly for steroidal sapogenins. Consequently, the search for these valuable precursors for cortisone and sex hormones has been conducted at a greatly increased rate.

IN RECENT years steroidal sapogenins have attracted considerable attention as precursors for sex and cortical hormones (*3–5*). This laboratory has been engaged for some time in a comprehensive survey of the plant kingdom in an attempt to find good sapogenin sources.

The sapogenins are not found free in nature, but occur in a combined glycosidal form called saponins (*1*). Steroidal saponins are not widespread. Many plants contain little or no saponin; in others the triterpenoid saponins predominate. Because the methods available for large scale isolation and identification of steroidal sapogenins are tedious (*4, 6*), a procedure which would rapidly eliminate samples containing little or no steroidal sapogenin would be useful. Although numerous reports on hemolytic methods for "saponins" have been published, a literature search showed that no specific method was available (*1*).

A rapid microprocedure is reported here. Saponins are detected by hemolytic methods (*1*). If the sample gives a positive test, the saponins are hydrolyzed, and steroidal sapogenins in the crude mixture are detected and estimated by means of their specific infrared absorption spectra.

PROCEDURE

Extraction. WET SAMPLES. A 400- to 500-gram sample of wet plant tissue is put through a Ball and Jewell grinder with a 1-inch screen. Two hundred grams of the ground tissue are covered with approximately 500 ml. of 95% ethyl alcohol and refluxed for 15 to 30 minutes. The sample is then cooled, filtered, washed, and made to a volume of 1 liter with 95% ethyl alcohol.

DRY SAMPLES. Fifty grams, ground to pass an 80- to 100-mesh screen, are covered with 300 ml. of 80% ethyl alcohol and refluxed for 1 hour. The sample is then treated the same as the wet sample, except that it is adjusted to a final volume of 500 ml. with 80% ethyl alcohol.

Much smaller quantities of both wet and dry samples can be used with proportionately smaller volumes of solvent. Sampling errors can be serious with small quantities of plant tissue, and hence larger samples should be used if available.

Hemolytic Detection of Saponins. BLOOD STANDARDIZATION. Ten to 20 ml. of whole human blood are suspended in 100 ml. of 0.85% aqueous sodium chloride solution, the suspension is centrifuged and the supernatant liquid is decanted. The process is repeated twice. The blood corpuscles are then suspended in 400 ml. of 0.85% sodium chloride solution. Ten milliliters of the turbid suspension and 1 ml. of digitonin solution (10 mg. of pure digitonin in 100 ml. of 80% ethyl alcohol) are mixed in a 15-ml. conical centrifuge tube. The mixture is kept at room temperature for 5 minutes, and then visually compared with a tube of untreated blood suspension. If complete hemolysis has occurred, the tube containing digitonin will be entirely clear. This can be checked by centrifuging. The hemolyzed solution should give little or no red corpuscle precipitate.

Within the authors' experience, complete hemolysis by 1 ml. of digitonin does not occur under these experimental conditions. Hence the stock blood suspension is progressively diluted with 0.85% sodium chloride solution until 10 ml. of the blood suspension are completely hemolyzed at room temperature within 5 minutes. In this manner, different blood samples can be roughly standardized to give equivalent hemolysis with digitonin. The stock solution can be kept for 1 to 2 weeks under refrigeration.

DETECTION OF SAPONINS. One milliliter of the plant extract described above is added to 10 ml. of standardized blood suspension. After 5 minutes, the presence or absence of hemolysis is observed as discussed under blood standardization. Samples giving a negative test are discarded. Positive extracts are used for detection of steroidal sapogenins.

Isolation of Crude Sapogenins. An aliquot of the alcoholic extract equivalent to 5.0 grams of original plant material (moisture-free basis) is evaporated just to dryness on a steam bath. The product is transferred with a total of 5 ml. of 50% ethyl alcohol to a reaction tube with an over-all length of 13.0 cm. and an outside diameter of 1.6 cm. The reaction tube is essentially a test tube provided with a ⅎ 14/35 outer joint.

Five milliliters of benzene saturated with 50% ethyl alcohol are added to the concentrated extract in the reaction tube. The layers are vigorously shaken, and the tube is centrifuged at 1500 to 2000 revolutions per minute for 1 minute. The benzene layer is drawn off, and the benzene extraction is repeated. The benzene extracts containing fats, pigments, etc., are discarded.

Sufficient concentrated hydrochloric acid is added to the residual extract to make the resultant acid concentration approximately 4 *N*. One or 2 ml. of benzene (alcohol-saturated) are added, and the tube is attached to a small condenser provided with a 14/35 ⅎ inner joint. The tube is immersed in a water bath kept at 78° to 80° C. for 2 hours. The tube is then withdrawn from the bath and cooled, 5 ml. of benzene containing 10% ethyl alcohol are added, and the layers are vigorously mixed. The tube is centrifuged, and the benzene layer withdrawn. The benzene extraction is repeated twice.

The combined benzene extracts containing crude sapogenin are placed in a small beaker and evaporated to dryness on the steam bath, 2 ml. of acetic anhydride are added, and the mixture is gently boiled for several minutes. The acetylated crude sapogenin is transferred to a 15-ml. centrifuge tube with 5 ml. of benzene, 5 ml. of methanol saturated with potassium hydroxide are added, and the contents are mixed vigorously. Immediately 5 ml. of water are added and mixed well, and the tube is centrifuged. The benzene layer which separates is withdrawn, and the residual aqueous methanol is twice re-extracted with benzene. The combined benzene layers containing the crude sapogenin acetate are placed in a tared beaker and evaporated to dryness on the steam bath. The beaker is then dried to constant weight in a vacuum oven at 110°.

Samples with a residue weight of less than 10 mg. are discarded. Samples between 10 and 100 mg. are dissolved in carbon disulfide, 5 ml. of carbon disulfide are added, and the beaker is cautiously warmed until complete solution of the crude acetate takes place. The solution is cooled and filtered through a microfilter into a 5-ml. volumetric flask. The filter is washed with carbon disulfide until the solution is up to volume. If the crude acetate weight is in excess of 100 mg., a larger volume (10 ml.) is used. Occasionally if a sample is difficultly soluble in carbon disulfide, chloroform is used as the solvent.

Infrared Determination. The infrared spectrum of the solu-

tion, relative to the pure solvent, is obtained from 800 to 1050 cm.$^{-1}$ (12.5 to 9.5 microns) in a 1-mm. cell and examined for the presence of the four characteristic sapogenin absorption bands. (A Beckman Model IR-3 infrared spectrophotometer was used in these studies.) Bands near 852, 900, 922, and 987 cm.$^{-1}$ (11.75, 11.1, 10.85, and 10.14 microns), with the 922 band stronger than the 900 band, indicate a "normal" sapogenin. Bands near 866, 900, 922, and 982 cm.$^{-1}$ (11.55, 11.1, 10.85, and 10.18 microns), with the 900 band stronger than the 922 band, indicate an "iso" sapogenin. If the bands at 900 and 922 cm.$^{-1}$ are not much different in strength and the remaining bands lie between the values given above, the sample contains sapogenins of both configurations. All four bands should be present before it is concluded that the sample contains significant amounts of sapogenin, because isolated bands of other substances can occur in this region of the spectrum.

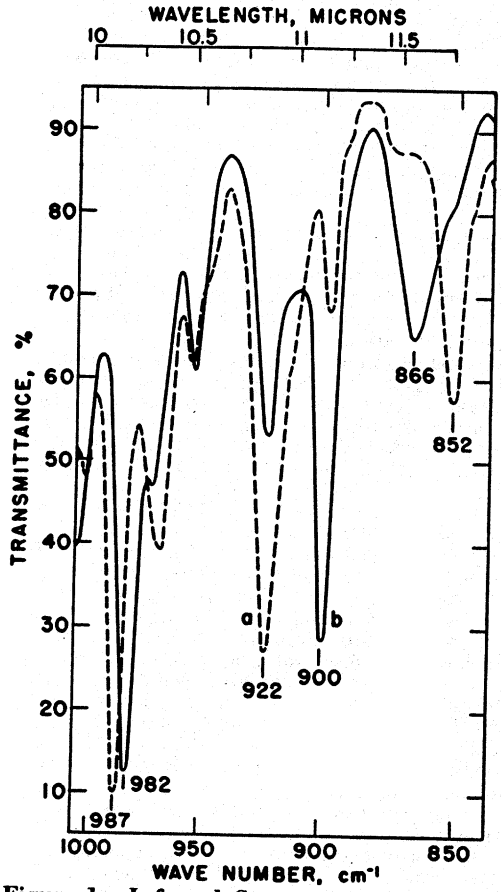


Figure 1. Infrared Spectra of Sarsasapogenin (*a*) and Smilagenin (*b*) Acetates

Concentration 10.0 grams per liter; solvent, carbon disulfide

For an approximate determination of the quantity of sapogenin in the sample, the 982–987 cm.$^{-1}$ absorption band is used, with a correction for background due to impurities and nearby absorption bands. A straight line is drawn between the two points of maximum transmittance on opposite sides of the 982–987 cm.$^{-1}$ band. Another straight line perpendicular to the frequency axis is drawn through the point of minimum transmittance of this band. The absorbance value at the intersection of these two straight lines is subtracted from the absorbance of the point of minimum transmittance, and the corrected absorptivity is calculated from this absorbance difference. [Absorptivity (or extinction coefficient) equals absorbance (or optical density) divided by concentration and by cell thickness.] In calculating the absorptivity, the concentration used is that of the equivalent amount of original plant material—for example, if the extract from 5 grams of plant material is contained in 5 ml. of final solution, the concentration is called 1000 grams per liter. If the sample is found to contain primarily "iso" sapogenins, the corrected absorptivity is divided by 0.53 liter per gram per centimeter (the corrected absorptivity of diosgenin acetate) to obtain the weight fraction of iso sapogenins in the original plant material. If the sample is found to contain primarily "normal" sapogenins, its corrected absorptivity is divided by 0.63 liter per gram per centimeter (the corrected absorptivity of sarsasapogenin acetate), to obtain the weight fraction of normal sapogenins in the original plant material. If the sample contains both types of sapogenins (rarely the case in the authors' experience), a rough estimate of sapogenin content is obtained by dividing the corrected absorptivity by 0.58 liter per gram per centimeter. Because the straight-line background correction, described above, cannot be an exact representation of the contribution of the nonsapogenin material to the absorption curve, this estimate of sapogenin content must be considered as only approximate if the plant extract contains substantial amounts of nonsteroidal materials.

As a numerical example, curve *a* of Figure 3 has an absorption band at 982 cm.$^{-1}$ with a transmittance of 22.3%. On opposite sides of this band are transmittance maxima at 970 and 997 cm.$^{-1}$ A straight line drawn between these two maxima crosses the 982 cm.$^{-1}$ coordinate at a transmittance of 66.3%.

$$\text{Corrected absorbance} = \log 66.3 - \log 22.3 = 0.473$$

Cell thickness was 0.109 cm. The extract from 5 grams (moisture-free basis) of original plant material had been dis-

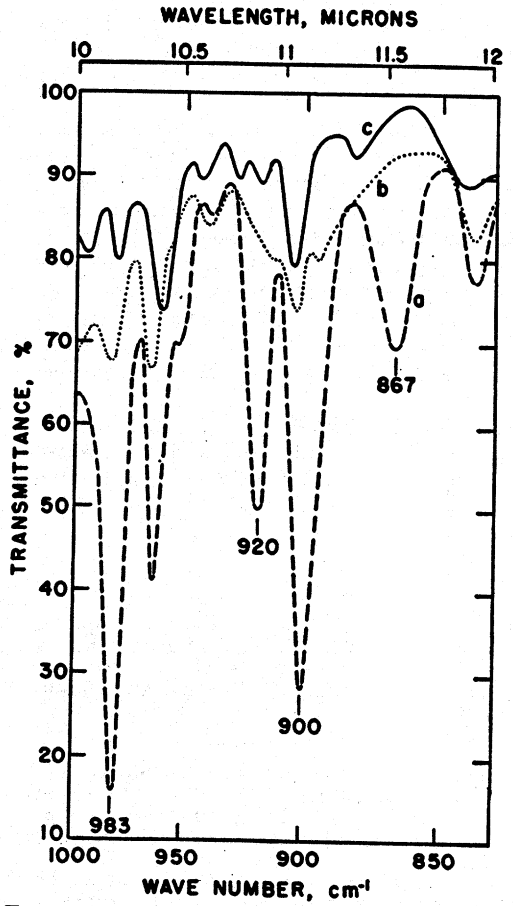


Figure 2. Infrared Spectra of Diosgenin (*a*), Dihydrodiosgenin (*b*), and Cholesterol (*c*) Acetates

Concentration approximately 10 grams per liter; solvent carbon disulfide

solved in 10 ml. of final carbon disulfide solution. Hence concentration = 5/0.010 = 500 grams per liter.

$$\text{Corrected absorptivity} = \frac{0.473}{0.109 \times 500} = 0.00868$$

Since the 900 cm.$^{-1}$ band is stronger than the 920 cm.$^{-1}$ band, this is an iso sample and should be compared with the reference absorptivity of 0.53 liter gram^{-1} cm.$^{-1}$

$$\text{Weight fraction} = \frac{0.00868}{0.53} = 0.016$$

Thus the original plant material contained 1.6% iso sapogenin, on a moisture-free basis, calculated as diosgenin acetate.

DISCUSSION

Detection and estimation of steroidal sapogenins in plant tissue are essentially a three-phase process: detection of the steroidal saponins which are the glycosidal precursors of the sapogenins, isolation of the crude sapogenin acetate, and infrared detection of steroidal sapogenins. These three phases are discussed in turn.

Detection of Saponins. Steroidal saponins are soluble in aqueous alcohols. Under experimental conditions, which are based on larger scale studies at this laboratory (*6*), 70 to 80% of the total saponin is extracted. Repeated alcohol extractions would undoubtedly recover the remainder. In view of the overall uncertainties in the procedure, prolonged extraction is not warranted for routine purposes.

Saponins are rapidly detected by their hemolytic activity (*1*). However, the test is not specific; triterpenoid saponins, which are widespread, are powerful hemolyzers. Undoubtedly other substances present in plant extracts give a positive test. Efforts to make the test specific by making assays before and after addition of cholesterol (*2*) were also fruitless, because in contrast to digitonin many other steroidal saponins do not form insoluble complexes with cholesterol. Until the screening procedure described in this paper was evolved, more than half the extracts which gave positive hemolysis tests and were put through the long large scale assay procedure (*6*) yielded no crystalline steroidal sapogenin.

On the other hand, a negative hemolysis test by the previously described procedure can be taken as a sensitive and specific test for the absence of saponins. More than 50 plant samples which gave a negative test were examined by the large scale procedure. Included among the samples were many different species. In no case could an appreciable quantity of steroidal sapogenin be isolated. Other tests of these nonhemolyzing samples, using the more sensitive infrared estimation, have shown a similar absence of significant amounts of steroidal sapogenin.

Isolation of Crude Steroidal Sapogenins. The procedure used is essentially a modification of the large scale one used in this laboratory (*6*).

In brief, the concentrated saponin solution is defatted and then acid-hydrolyzed to give the steroidal sapogenin, which, like most steroids, is benzene-soluble and water-insoluble. The sapogenin is converted to the acetate to increase its solubility in carbon disulfide for the infrared examination. The crude acetate is treated with alkali to remove excess acetic anhydride and many pigmented phenolic compounds produced by acid hydrolysis of plant extracts. The alkali is rapidly removed from the sample to minimize hydrolysis of the acetate. Tests with pure sapogenins subjected to this procedure resulted in excellent recoveries (90 to 95%).

In many cases the positive hemolysis reaction is given by substances subsequently removed during the isolation of the crude sapogenin acetate—for example, many carboxylic triterpenoid saponins. In such samples there is little or no residue of crude

Table I. Characteristic Sapogenin Absorption Bands

Sapogenin Acetate	Wave Length, μ	Wave No., Cm.$^{-1}$	Molar Absorptivity[a], L. Mole^{-1} Cm.$^{-1}$
Chlorogenin	11.55	866	82.5
	11.11	900	285
	10.86	921	103
	10.17	983	365
Diosgenin	11.54	867	66.3
	11.11	900	229
	10.87	920	126
	10.17	983	335
Gitogenin	11.53	867	72.2
	11.11	900	254
	10.81	925	130
	10.18	982	403
Hecogenin	11.53	867	73.7
	11.11	900	284
	10.86	921	133
	10.18	982	352
Manogenin	11.53	867	68.1
	11.10	901	284
	10.86	921	139
	10.18	982	393
Samogenin	11.56	865	82.4
	11.11	900	206
	10.86	921	113
	10.18	982	393
Sarsasapogenin	11.74	852	102
	11.15	897	69.9
	10.85	922	239
	10.14	987	422
Smilagenin	11.55	866	78.7
	11.11	900	230
	10.84	922	116
	10.18	982	381
Tigogenin	11.56	865	72.4
	11.11	900	230
	10.82	924	140
	10.17	983	341

[a] Not corrected for background absorption.

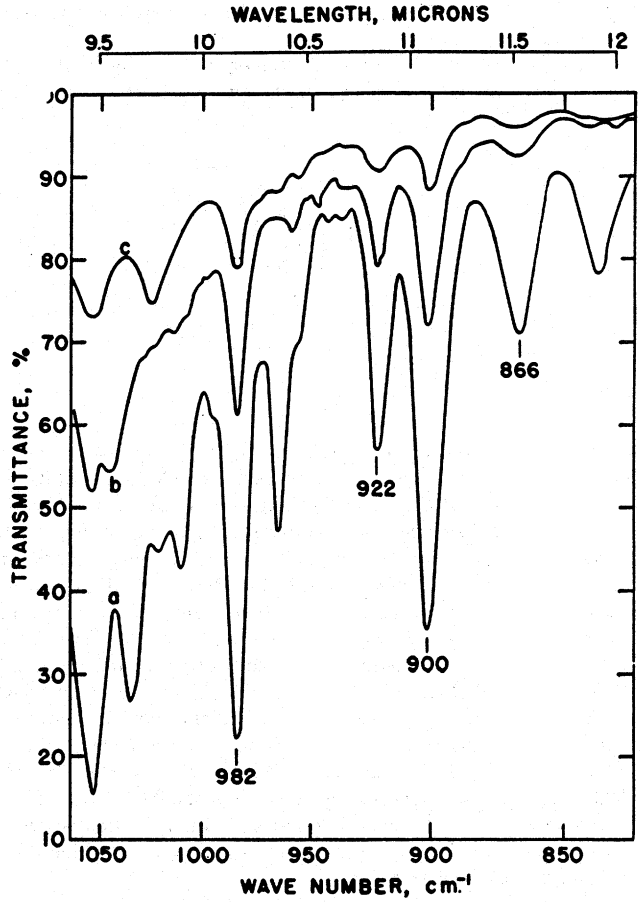
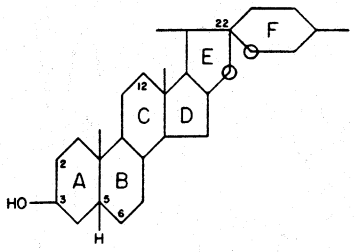


Figure 3. Infrared Spectra of Crude Iso Sapogenin Acetates

a. 1.6%
b. 0.2%
c. 0.08%

acetate, and hence they can be discarded without going through the infrared procedure.

Infrared Detection of Steroidal Sapogenins. The structure of a typical steroidal sapogenin, smilagenin (22-isospirostan-3β-ol) is shown below.



Typical variants are isomerism at C_5 or C_{22}, unsaturation between C_5 and C_6, presence of a carbonyl at C_{12}, and additional hydroxyl groups at C_2, C_6, or C_{12}. Saturated, noncarbonyl sapogenins lack any specific chemical characteristic useful for identification purposes, and their unsaturated or ketonic analogs are but slightly better in this respect. The infrared absorption spectra of sapogenin acetates, however, reveal some striking characteristics. As shown in Figures 1 and 2 and Table I, all iso sapogenins have characteristic absorption bands near 866, 900, 922, and 982 cm.$^{-1}$ (11.55, 11.1, 10.85, and 10.18 microns).

Sarsasapogenin (spirostan-3β-ol), which has a "normal" configuration, has bands at 852, 900, 922, and 987 cm.$^{-1}$ (11.75, 11.1, 10.85, and 10.14 microns). With normal sapogenins, the 922 band has a stronger absorptivity than the 900 band. In iso sapogenins this relationship is reversed. Figure 1 demonstrates this relationship for sarsasapogenin and smilagenin, which differ only in the configuration at C_{22}.

The four characteristic infrared absorption bands are apparently a property of ring F. This is demonstrated by Figure 2, which gives the infrared absorption of the acetates of diosgenin (Δ^5-22-isospirosten-3β-ol), dihydrodiosgenin (Δ^5-22-furosten-3β-26-diol), and cholesterol (Δ^5-cholesten-3β-ol). These compounds have identical structures in rings A, B, C, and D. Cholesterol differs from diosgenin in that a saturated hydrocarbon side chain is attached to ring D in place of rings E and F; dihydrodiosgenin retains rings A, B, C, D and E, but ring F is opened. It should be noted from Figure 2 that only diosgenin has all four typical sapogenin absorption bands. Several other dihydrosapogenins were tested and all lack the typical four sapogenin bands. The presence of this system of four absorption bands is positive qualitative evidence of the presence of steroidal sapogenins in the plant

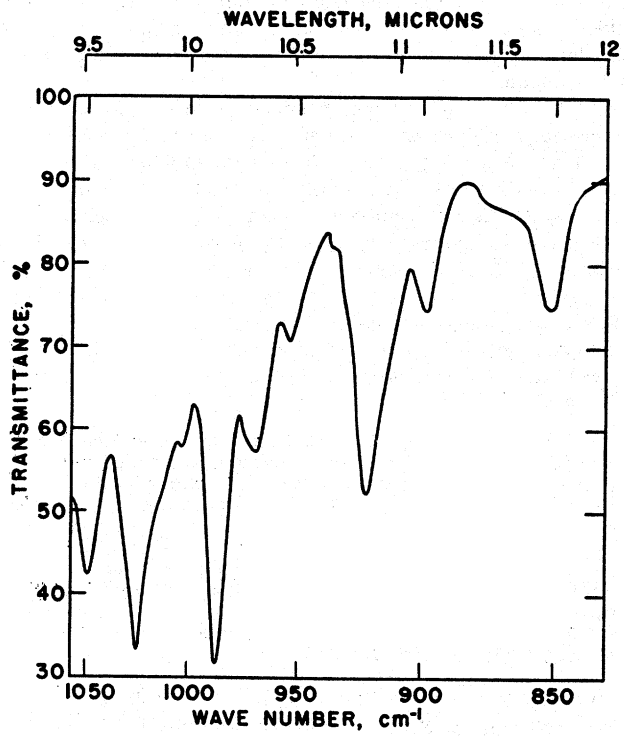


Figure 4. Infrared Spectrum of Crude Normal Sapogenin Acetate, 0.45%

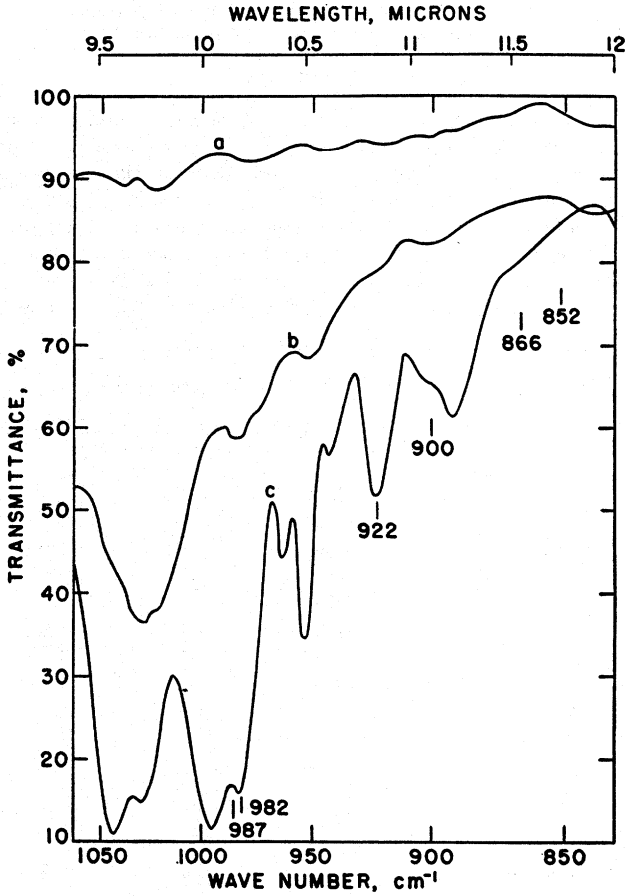


Figure 5. Infrared Spectra of Nonsteroidal Substances

sample. The four characteristic sapogenin bands do not depend upon the presence of the acetate group. The free sapogenins themselves also show these four bands.

From the data it is apparent that the characteristic infrared sapogenin absorption is due to the presence of ring F. No other known naturally occurring compounds have the sapogenin ring structure. Hence the method is specific.

The bands show up in a clear-cut manner in the crude sapogenin preparations. The wave lengths are exactly the same as those of the pure reference compounds. The absorption spectra of most crude preparations indicate whether the sapogenin is iso or normal. Because sarsasapogenin is the only common normal sapogenin, the absorption spectrum is a fairly good test for the presence or absence of this compound.

Figures 3, 4, and 5 show some typical infrared absorption curves that indicate positive and negative tests for sapogenins. Curves *a*, *b*, and *c* (Figure 3) are iso sapogenins; Figure 3, *a*, is a high sample (1.6% moisture free basis), *b* intermediate (0.2%), and *c* low (0.08%). The typical sapogenin peaks are clear even with low samples such as Figure 3, *c*. Figure 4, *a*, shows a similar normal sapogenin (0.45% moisture-free basis).

Curves *a*, *b*, and *c* (Figure 5) show typical spectra for plant extracts containing no steroidal sapogenins. Index marks show

the wave numbers at which the iso and normal sapogenin band systems should occur. It can be seen that none of them possess the characteristic system of four sapogenin absorption bands, although some contain one or two bands at nearly the same frequencies. Curve *c* is the spectrum of a triterpenoid sapogenin. Such a compound could not be mistaken for a steroidal sapogenin because it does not have the entire system of four bands. However, if a substantial amount of such substance occurs mixed with a small amount of steroidal sapogenin in a plant extract, it can lead to an erroneously high estimate of the amount of steroidal sapogenins because of its absorption at 982 cm.$^{-1}$ Certain other substances found in plant materials also have weak absorption bands near 982–987 cm.$^{-1}$ but do not have the complete system of four sapogenin bands.

Therefore, although the infrared method appears to give positive evidence of the presence or absence of steroidal sapogenins, the estimate of percentage of sapogenin is only semiquantitative if considerable amounts of nonsteroidal material are present. Nevertheless, it is the only method now available which will give even an approximate assay on a crude material. Two workers, one carrying out the hemolysis and crude sapogenin isolation steps and the other the infrared procedure, can handle 40 to 50 samples in a 40-hour week.

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LITERATURE CITED

(1) Kofler, L., "Die Saponine," Vienna, J. Springer, 1927.
(2) Kofler, L., and Raum, H., *Biochem. Z.*, **219**, 335 (1930).
(3) Marker, R. E., and Applezweig, N., *Chem. Eng. News*, **27**, 3348 (1949).
(4) Marker, R. E., Wagner, R. B., Ulshafer, P. R., Wittbecker, E. L., Goldsmith, D. P. J., and Ruof, C. H., *J. Am. Chem. Soc.*, **69**, 2167 (1947).
(5) Rosenkranz, G., Pataki, J., and Djerassi, C., *Ibid.*, **73**, 4055 (1951).
(6) Wall, M. E., Krider, M. M., Rothman, E. S., and Eddy, C. R., *J. Biol. Chem.*, in press.

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